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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of:

JACOBS ET AL.

Serial Number: 09/544,683   Group Art Unit: To be assigned

Filed: April 7, 2000   Examiner: to be assigned

For: CAMPYLOBACTER VACCINE

<u>CLAIM TO PRIORITY UNDER 35 USC 119</u>

Assistant Commissioner of Patents   April 20, 2000
Washington, D.C. 20231

Sir:

   The benefit of the filing date of the following prior foreign application is hereby requested for the above-identified application, and the priority provided in 35 USC 119 is hereby claimed:

 European Patent Application No. 99201086.8, filed April 9, 1999

   In support of this claim, the requisite certified copy of said original foreign application is filed herewith along with a verified English translation thereof.

   It is requested that the file of this application be marked to indicate that the Applicant has complied with the requirements of 35 USC 119 and that the Patent and Trademark Office kindly acknowledge receipt of this document.

   In the event any fees are required with this paper, please charge our Deposit Account No. 02-2334.

                              Respectfully submitted,

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Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.	The attached documents are exact copies of the European patent application described on the following page, as originally filed.	Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr.   **Patent application No.**   **Demande de brevet n°**

99201086. 8

Der Präsident des Europäischen Patentamts:
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
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Blatt 2 der Bescheinigung
Sheet 2 of the certificate
Page 2 de l'attestation

Anmeldung Nr.:
Application no.: **99201086.8**
Demande n°:

Anmeldetag:
Date of filing: **09/04/99**
Date de dépôt:

Anmelder:
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NETHERLANDS

Bezeichnung der Erfindung:
Title of the invention:
Titre de l'invention:

Campylobacter vaccine

In Anspruch genommene Prioriät(en) / Priority(ies) claimed / Priorité(s) revendiquée(s)

Staat: State: Pays:	Tag: Date: Date:	Aktenzeichen: File no. Numéro de dépôt:

Internationale Patentklassifikation:
International Patent classification:
Classification internationale des brevets:

A61K39/40, C07K14/205

Am Anmeldetag benannte Vertragstaaten:
Contracting states designated at date of filing: AT/BE/CH/CY/DE/DK/ES/FI/FR/GB/GR/IE/IT/LI/LU/MC/NL/PT/SE
Etats contractants désignés lors du depôt:

Bemerkungen:
Remarks:
Remarques:

Campylobacter vaccine

The present invention relates to vaccines against *Campylobacter* colonisation, to the use of *Campylobacter* proteins and anti-*Campylobacter* antibodies for the preparation of such vaccines and to methods for the preparation of such vaccines.

Bacteria of the genus *Campylobacter* are Gram-negative spiral shaped pathogenic bacteria, with a high motility and carrying a flagellum at one or both poles of the cell. Several *Campylobacter* species have been found. *Campylobacter jejuni* is very often found in poultry. Frequently *Campylobacter coli* and (to a lesser extent) the recently found *Campylobacter hyoilei* are found in pigs.

Of these, *Campylobacter jejuni* is the most frequently isolated *Campylobacter* species in association with human diarrhoea. It is becoming more and more evident that the number of *Campylobacter* infections in humans exceeds the number of Salmonella infections. (Griffiths et al., Joum. of Applied Bacteriology 1990, 69: 281-301, Walker et al., Microbiological reviews 1986, 50: 81-94, Butzler, J-P., ISBN 0-8493-5446-3, RIVM Report No. 216852002, Bilthoven, the Netherlands). It is difficult to avoid infection in humans with *Campylobacter* since, first of all, *Campylobacter* is a food borne zoonotic bacterium for which many animals, both wild and domestic, healthy or sick serve as a reservoir. In addition the bacterium has many different routes of transmission. Bacteria can survive in a dormant coccal form for several weeks on e.g. the surface of carcasses and in water. The bacterium can therefore easily be transmitted to man through direct contact with animals or by means of contaminated water or food, e.g. milk or meat. *C. jejuni* is present in many healthy animals, e.g. avian species such as turkey and chicken, cattle, sheep, horses and rodents. Chicken meat, an important nutrient source in many countries world-wide is known to be very frequently contaminated with *Campylobacter* (Shane (1992), S.M., Avian Pathology 21: 189-213). This is not only the case in developing countries but also in e.g. Europe. *Campylobacter* resides in the gut of poultry. Contamination of the meat frequently happens in the slaughterhouse when the intestinal tract, which is often heavily *Campylobacter*-contaminated, is removed from the animal. Contamination during slaughter is very difficult to avoid. In the Netherlands, about 50 % of the chicken meat is contaminated, in spite of the high hygienic standards applied in meat industry. A recent overview of the Epidemiology of *Campylobacter* in poultry is given in the Thesis of C.M. Karssen, (ISBN 90-71463-72-

9). As a result of this high contamination pressure, about 300,000 persons annually in th Netherlands only (total population 15.000.000) suffer from *Campylobacter* infection, caused by handling or eating undercooked poultry meat. These figures are not significantly different in other European countries. World-wide, annually more than 400.000.000 cases are estimated to occur (Pace et al., Vaccine 1998, 16: 1563-1574). *Campylobacter* causes enteric infections in humans, and occasionally more severe diseases like abortion, meningitis, apendicitis, and urinary tract infection. (Blaser et al., New Engl. J. Med. 1981, 305: 1444-1452, Butzler et al., Clinics in Gastroenterol. 1979, 8: 737-765). Also, severe neurologic complications such as Guillain-Barré syndrome and Miller-Fisher syndrome are sometimes seen (Schwerer et al., 1995, J. Endotox. Res. 2: 395-403 and Salloway et al., 1996, Infect. Immun. 64: 2945-2949). Diarrhoea due to *Campylobacter jejuni* is usually a self-limiting infection, lasting about 2-7 days. In young children, old people and immunocompromised patients, the disease is not self-limiting and requires antibiotic treatment. It is clear that, if a potential vaccine against *Campylobacter* for human use would be available, it could prevent humans from becoming infected. This would however require a standard vaccination comparable to vaccination against e.g. mumps and measles. This is evidently not practical. A more logical approach lies in avoiding the transmission from animal to man, specifically from poultry to man. The easiest way of doing this is by vaccinating poultry against *Campylobacter* infection. Vaccination of poultry (as well as human vaccination) has however turned out to be much more complicated than was initially expected. This is due to the fact that *Campylobacter* is, in spite of the fact that it colonises the gut, not pathogenic to poultry. Most vaccines tested are inactivated whole cell preparations, administered systemically or orally, sometimes in combination with adjuvants. In some cases colonisation of the gut could, to a certain extent, be decreased, but there are no examples of vaccines avoiding colonisation. Shedding of *Campylobacter* could not be stopped by any of these vaccines. Killed whole cell vaccines, if compared with subunit vaccines, have been considered the best candidates for a vaccine, because in principle they still possess all potential immunogenic determinants. Next to the development of whole cell vaccines, much effort has been put in the development of flagella-based subunit vaccines. Flagella have been recognised as the immunodominant antigen recognised during infection and numerous studies have suggested a role for this protein in protection (Martin et al., Inf. And Immun. 1989, 57: 2542-2546, Wenman et al., J. Clin.

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Microbiol. 1985, 21: 108-112). Flagella-less mutants are known not to colonise the gut, and they disappear from the infected animal within one or two weeks whereas the wild type bacterium remains present in the gut. Flagella are thus by far the most likely candidates for the preparation of a vaccine, especially since they seem to play a key role, if not the only role, in the colonisation of the gut. If colonisation could be prevented, that would be a first step in the elimination of contamination in poultry. Nevertheless, potential vaccines based upon the flagella of *Campylobacter* have not given an acceptable level of protection.

Next to active vaccination as described above, passive vaccination has been tested as a means of decreasing *Campylobacter* infection. Tsubokura et al (1997, Clin. Exp. Immunol. 108: 451-455) have orally administered antibodies against whole *Campylobacter jejuni* cells followed by challenge with *Campylobacter jejuni*. They claim a 1-2 log reduction in the number of bacteria found in the faeces of thus vaccinated chickens. All efforts made so far have not yet led to any vaccine, be it live, inactivated or on the basis of subunits, that is capable of significantly diminishing the level of colonisation and the amount of bacteria shedded in the faeces. It is clear, that there still is a need for a reliable and safe vaccine or alternative treatment.

In principle, there is no need to protect poultry against *Campylobacter* infection during their whole life span. They do not suffer from infection as explained above. Therefore, a treatment capable of diminishing the amount of bacteria and thus the infective pressure shortly before slaughter would be an efficient treatment for suppressing subsequent contamination of the meat during slaughter. And this in turn would prevent meat-transmitted human *Campylobacter* infection.

It is an objective of the present invention to provide a vaccine that is capable of both diminishing the level of colonisation and shedding, or even of eliminating *Campylobacter* from the caecum of poultry. This avoids *Campylobacter*-contamination of the meat during slaughtering and therefore avoids subsequent infection of humans.

It was surprisingly found now that a vaccine having these characteristics can be based upon antibodies against flagella-less mutants of *Campylobacter*. This is highly unexpected since, as mentioned above, flagella are considered to be the key protein involved in adherence and colonisation. Even more surprising, such a vaccine does diminish colonisation and shedding of wild type flagellated *Campylobacter*. This is the

first tim that a vaccine is reported that is even capabl to eliminat *Campylobact r* from the ceca below the level of detection.

Thus, one embodiment of the invention relates to vaccines for the prevention of *Campylobacter* colonisation in animals, which vaccines comprise antiserum against a flagellaless *Campylobacter* strain.

Such a vaccine can in a very simple form comprise just isolated antiserum against *Campylobacter* and possibly a diluent. Such a diluent can be added to dilute the antiserum if the amount of antibody titre is too high. The diluent can be as simple as distilled water, or physiological salt solution. Actually any pharmaceutically acceptable diluent can be used.

The invention is equally applicable for *Campylobacter* contamination in poultry, pigs and other animals.

Given however the very high contamination pressure of chicken meat, a preferred form of this embodiment relates to flagellaless *Campylobacter* strains of the species *Campylobacter jejuni*, and to poultry.

Any flagella-less *Campylobacter* strain can be used for raising antiserum. Especially those flagella-less *Campylobacter* strains that have growth-rates comparable to wild type strains are preferred. A very suitable flagella-less *Campylobacter* strain for raising antibodies has been described by Wassenaar, T.M., Bleumink-Pluym, N.M.C. and van der Zeijst, B.A.M. 1991, in the EMBO Journal 10:2055-2061.

Thus, in a preferred form, the flagella-less *Campylobacter jejuni* strain against which the antibodies are raised, is strain R2.

Antibodies suitable for use in a vaccine according to the present invention can be obtained from polyclonal sera, monospecific sera or from monoclonal antibody culture. Polyclonal sera have the advantage that they are easily made according to standard techniques. Techniques for producing and processing polyclonal sera are abundantly known in the art (e.g. Mayer and Walter, eds. Immunochemical Methods in Cell and Molecular Biology, Academic Press, London, 1987). Animals suitable for raising the antibodies are e.g. cows, rabbits, mice and chickens. Efficient methods for obtaining bovine antibodies against *Campylobacter* are d scribed by Hilp rt et al., 1987, J. Inf.

Diseases 156: 158-166. Anoth r attractive way of producing large amounts of anti-bodies i.e. production in gg yolk has been described by Hatta et al., 1993, Biosci. Biotech. Biochem. 57: 450-454.

Another embodiment of the present invention relates to the use of antibodies against a flagella-less *Campylobacter jejuni* strain for the preparation of a vaccine against *Campylobacter jejuni* colonisation.

Even more surprisingly, the following was found: antiserum raised against flagellaless *Campylobacter jejuni* mutants recognises two major protein bands, a 97 kD (+/- 5 kD) and a 60 kD (+/- 5 kD) band, on a Western blot of total *Campylobacter jejuni* protein, that are not seen when using antiserum against wild type *Campylobacter jejuni*. This phenomenon is equally seen in Western blots of wild type *Campylobacter* and of flag-ellaless *Campylobacter*. Thus, the two proteins are equally present in wild type and flagellaless *Campylobacter* strains. It was therefore concluded that the recognition of these specific proteins by the immune system only occurs on the absence of the fla-gella.

As mentioned above, antiserum against flagellaless *Campylobacter* mutants is capable of eliminating *Campylobacter* from the ceca below the level of detection. This antise-rum differs from antiserum against wild type Campylobacter (not capable of eliminating *Campylobacter*) in that it additionally comprises antibodies against the 97 kD and 60 kD proteins. These two proteins apparently only induce antibodies if the flagella is ab-sent, so it was concluded that these two proteins are capable of inducing antibodies that play an essential role in the elimination of *Campylobacter* from the ceca. There-fore, antibodies raised against either the 97 kD or the 60 kD protein or a combination thereof are equally capable of eliminating *Campylobacter* strains from the ceca.

Thus another embodiment of the invention relates to antigenic proteins having a mo-lecular weight of 97 kD or 60 kD, that are visible in a Western blot of *Campylobacter jejuni* protein after incubation of that Western blot with antibodies against a flagellaless mutant of *Campylobacter jejuni* and that are not visible after incubation of that blot with antibodies against wild type *Campylobacter jejuni*.

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The 97 kD or the 60 kD proteins can be used to produce antibodies, which may be polyclonal, monospecific or monoclonal (or d rivatives thereof). Th 97 kD and 60 kD protein can be isolated according to many standard protein isolation procedures well-known in the art. One very easy method is the excision of these proteins from a preparative gel. If polyclonal antibodies are desired, techniques for producing and process-ing polyclonal sera are known in the art (e.g. Mayer and Walter, eds., see above). Monoclonal antibodies, reactive against the 97 kD or the 60 kD proteins according to the invention (or variants or fragments thereof) according to the present invention, can be prepared by immunising inbred mice by techniques known in the art (Kohler and Milstein, *Nature*, 256, 495-497, 1975).

One of the advantages of using antibodies against any of these two proteins instead of antiserum against the whole flagellaless *Campylobacter* is, that specific monoclonal antibodies against these two proteins can easily be obtained from large scale hybri-doma growth in fermentors. This allows the production of large amounts of antibodies at low costs/efforts and without the use of animals.

Thus, another embodiment of the invention relates to vaccines comprising antibodies against the 97 kD or the 60 kD proteins of *Campylobacter*.

Even still another embodiment of the present invention relates to the use of antibodies against the 97 kD and/or 60 kD protein according to the invention for the preparation of a vaccine against *Campylobacter jejuni* colonisation.

The methods for the preparation of a vaccine according to the invention need not be complex. In principle, it suffices to raise antibodies against a flagella-less mutant or against the 97 kD and/or 60 kD protein in e.g. an animal, followed by collecting the blood and isolating the antiserum according to standard techniques. Suitable animals for raising such antibodies are e.g. rabbits and chickens. When chickens are used, an-tibodies can alternatively be obtained from the egg yolk of systemically immunised chickens. In principle the antibodies need not be diluted. They can be given as such, or if necessary even in a concentrated form. Alternatively, if the antibody concentration is very high, the thus obtained antiserum can e.g. be diluted before administration.

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Thus, another embodiment of the present invention relates to methods for the preparation of a vaccine according to the invention. Such methods comprise raising antibodies against antigenic material of a flagellaless *Campylobacter jejuni* strain in a host animal followed by isolating the antibodies. In principle, this method will usually comprise the taking of blood from the host animal followed by purifying the antiserum, e.g. by centrifugation or filtration.

It is also possible to obtain cells that produce the desired antibodies, directed against the 97 kD and 60 kD proteins and to grow these in e.g. a fermentor. Antibodies can be harvested afterwards and they can be mixed, if necessary, with a pharmaceutically acceptable carrier. The advantage of such a method is that no animals need to be used for the preparation of the antibodies.

The invention is i.a. very suitable for treating broiler chicken, before slaughtering. Such broilers are usually slaughtered at six weeks of age. Therefore, treatment of the animals with the vaccine according to the invention at about one week before they are slaughtered causes a significant decrease in the level of *Campylobacter*-contamination. It is clear that the amount of vaccine to be given depends highly on the concentration of antibodies in the vaccine. As an indication may serve that very suitable amounts of antibodies are present in between 0.1 and 1 ml of crude antiserum. Antibody-titres in e.g. egg-yolk preparations can easily be determined by standard techniques well-known in the art, such as the ELISA technique.

The antibodies can easily be given as a rather crude preparation. A possible way of administration is e.g. feeding crude antiserum to chickens. Alternative routes of administration are e.g. admixing the serum with drinking water. Also, antibodies can be mixed directly with chicken food. For such purposes, an alternative is freeze-drying of the antibodies, thus enhancing their long term stability, before mixing them with the food or water. Also, the antibodies can be encapsulated before adding them to chicken food.

Still anoth r embodiment of the invention provid s an alternative for using antiserum or antibodi s according to the invention for vaccination purposes. Alt mativ ly, it is pos-

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sible to use the 97 kD or 60 kD protein according to the invention directly for vaccination purposes. Wh n the 97 kD or 60 kD protein are administered directly to poultry, they induce antibodies against the 97 kD or 60 kD protein directly. The animals then produce their own protective antibodies against *Campylobacter*. Again, this is surprising: administration of whole wild type *Campylobacter* strains does not lead to elimination of *Campylobacter* from the ceca, because wild type *Campylobacter* strains suppress the induction of antibodies against the 97 kD and 60 kD proteins. Thus, when the 97 kD and 60 kD protein are not given in an isolated form, but as a part of the whole wild type *Campylobacter* cell, they have no effect.

Thus, another embodiment relates to vaccines comprising the specific antigenic 97 kD or 60 kD protein that is visible in a Western blot of *Campylobacter jejuni* protein after incubation of that Western blot with antibodies against a flagellaless mutant of *Campylobacter jejuni* and that is not visible after incubation of that blot with antibodies against wild type *Campylobacter jejuni*.

Such a vaccine can easily be prepared by admixing the protein with a pharmaceutically acceptable carrier. A pharmaceutically acceptable carrier is understood to be a compound that does not adversely effect the health of the animal to be vaccinated, at least not to the extend that the adverse effect is worse than the effects seen when the animal is not vaccinated. A pharmaceutically acceptable carrier can be e.g. sterile water or a sterile physiological salt solution. In a more complex for, the carrier can e.g. be a buffer.

Suitable amounts of protein for the preparation of vaccines vary according to the way of administration. For systemic application, amounts between 1 and 1000 μg are very suitable. In vaccines for oral administration the amount may also be in this range. If however oral vaccination through drinking water is envisaged, possibly larger amounts of protein have to be given, due to spillage of water.

The vaccine according to the present invention may in a preferred presentation also contain an adjuvant. Adjuvants in general comprise substances that boost the immune response of the host in a non-specific manner. A number of different adjuvants are known in the art. Examples of adjuvants are Freunds Complet and Incomplete adju-

vant, vitamin E, non-ionic block polymers and polyamines such as dextransulphate, carbopol and pyran. Also very suitable are surface active substances such as Span, Tween, hexadecylamine, lysolecitin, methoxyhexadecylglycerol and saponins (i.a. Quil A[R]. Furthermore, peptides such as muramyldipeptides, dimethylglycine, tuftsin, are often used. Next to these adjuvants, Immune-stimulating Complexes (ISCOMS), mineral oil e.g. Bayol[R] or Markol[R], vegetable oils or emulsions thereof and Diluvac[R] Forte can advantageously be used. The vaccine may also comprise a so-called "vehicle". A vehicle is a compound to which the polypeptide adheres, without being co-valently bound to it. Often used vehicle compounds are e.g. aluminium hydroxide, -phosphate, sulphate or -oxide, silica, Kaolin, and Bentonite. A special form of such a vehicle, in which the antigen is partially embedded in the vehicle, is the so-called IS-COM (EP 109.942, EP 180.564, EP 242.380).

Often, the vaccine is mixed with stabilisers, e.g. to protect degradation-prone polypep-tides from being degraded, to enhance the shelf-life of the vaccine, or to improve freeze-drying efficiency. Useful stabilisers are i.a. SPGA (Bovarnik et al; J. Bacteriol-ogy 59: 509 (1950)), skimmed milk, gelatin, bovine serum albumin, carbohydrates e.g. sorbitol, mannitol, trehalose, starch, sucrose, dextran or glucose, proteins such as al-bumin or casein or degradation products thereof, and buffers, such as alkali metal phosphates.

Freeze-drying is an efficient method for conservation. Freeze-dried material can be stored stable for many years. Storage temperatures for freeze-dried material may well be above zero degrees, without being detrimental to the material.

Freeze-drying can be done according to all well-known standard freeze-drying proce-dures.

Vaccines comprising the 97 kD or 60 kD protein are preferably administered mucos-ally. This can e.g. by done by oral administration, through admixing of the vaccine with drinking water. Especially for poultry, additional methods such as intra-ocular vaccina-tion and intranasal vaccination are also very suitable ways of mucosal vaccination.

EXAMPLES

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Example 1: detection of a 97 kD and a 60 kD protein.

Bacterial strains:

Wild type 81116: *Campylobacter jejuni*, wild type, human isolate, flagellum phenotype A^+B^+, motile and invasive in vitro (Wassenaar, T.M., Bleumink-Pluym, N.M.C. and van der Zeijst, B.A.M. 1991, EMBO Journal 10:2055-2061).

Mutant 81116-R2: FlaA and flaB deletion mutant (+kanamycin insert), flagellum phenotype A^-B^-, not motile and not invasive in vitro (Wassenaar, T.M., Bleumink-Pluym, N.M.C. and van der Zeijst, B.A.M. 1991, EMBO Journal 10:2055-2061).

Cell growth: Strain *Campylobacter jejuni* 81116 was inoculated on Blaser Campylobacter agar and strain *Campylobacter jejuni* 81116-R2 was inoculated on Blaser Campylobacter agar + 40 µg/ml kanamycin. Plates were incubated for 48 hours at 41 degrees C under microaerophilic conditions. A small number of colonies from the agar plates were inoculated in Brucella broth + 1% yeast extract for strain 81116 and in Brucella broth + 1% yeast extract + 40 µg/ml kanamycin for strain 81116-2R. After incubation for 24 hours at 41 degrees C under microaerophilic conditions, cultures were checked for the total number of bacteria and 0.2% formalin was added for inactivation (room temperature for 24 hours). Inactivated bacteria were collected by centrifugation. Cell pellets were resuspended in 0.01 M Tris pH 7.4 to a protein concentration of 1.0 mg/ml. Then 20 µl of this suspension was added per slot and run, on NuPage gel 4-12% Bis-Tris. Western blotting was done with NuPage transfer buffer/10% methanol.

Preparation of chicken antisera.

4-Weeks-old chickens were IM vaccinated with 1 ml whole cell vaccine (see below) of strain 81116 or 81116-R2. One group of chickens was left unvaccinated. At 4 weeks after vaccination all chickens were bled to death. Sera were pooled per group and used for immunisation of 4-days-old chickens.

Sera thus obtained were diluted 20, 200 or 400 times and incubated with the Western blot according to standard methods.

<u>Results:</u> The left Western bl t (Fig. 1) comprises total antigen of wild type *Campylo-bacter* strain 81116, th right Western blot comprises antigen of flagellaless *Campylo-bacter* strain 81116-2R. Lanes 1 and 7, lanes 2 and 8, and lanes 3 and 9 were incubated with 20, 200 or 400 times diluted antiserum against wild type *Campylobacter* strain 81116 respectively. Lanes 4 and 10, lanes 5 and 11, and lanes 6 and 12 were incubated with 20, 200 or 400 times diluted antiserum against flagellaless *Campylo-bacter* strain 81116-2R respectively.

As can be clearly seen in lanes 4-6 and lanes 10-12, two bands are visible with molecular weights of 97 and 60 kD respectively, that are not visible in lanes 1-3 and 7-9. (the somewhat fainter and more diffuse bands in between these lanes are molecular weight markers).

This proves that flagellaless *Campylobacter* strains are capable of inducing an immune response against a 97 kD and a 60 kD protein, whereas wild type *Campylobacter* strains do not show this phenomenon.

Example 2: preparation of vaccines.

Bacterial strains:
Wild type 81116: see above.
Mutant 81116-R2: see above.

Preparation of chicken antisera for passive immunisation.
4-Weeks-old chickens were IM vaccinated with 1 ml whole cell vaccine (see below) of strain 81116 or 81116-R2. One group of chickens was left unvaccinated. At 4 weeks after vaccination all chickens were bled to death. Sera were pooled per group and used for immunisation of 4-days-old chickens.

Preparation of inactivated whole cell vaccines.
Strain *Campylobacter jejuni* 81116 was inoculated on Blaser *Campylobacter* agar and strain *Campylobacter jejuni* 81116-R2 was inoculated on Blaser *Campylobacter* agar + 40 µg/ml kanamycin. Plates were incubated for 48 hours at 41°C under microaerophilic conditions. Colonies from th agar plates w re inoculated in Brucella broth + 1% yeast xtract for strain 81116 and in Brucella broth + 1% yeast extract + 40 µg/ml kanamycin

for strain R2. After incubation for 24 hours at 41°C und r microaerophilic conditions, cultures wer check d for the total number of bacteria and 0.2% formalin was added for inactivation (room temperature during 24 hours). Inactivated bacteria were collected by centrifugation, suspended in PBS and used for vaccine preparation by mixing cells and a Freunds Incomplete type of water in oil emulsion. The vaccine emulsions contain approximately 10^9 bacteria per ml.

Preparation of *Campylobacter* challenge strain.

Strain *Campylobacter jejuni* 81116 was grown on Blaser *Campylobacter* agar plates at 41°C for 48 hours under microaerophilic conditions. The growth of one plate was suspended in Brucella broth + 1% Yeast extract and incubated at 41°C for 48 hours in a closed bottle. Chickens were challenged orally with 0.2 ml culture. Viability count of the challenge culture was determined by plate counting.

Example 3: vaccination experiments.

In this experiment, a comparison is made between passive vaccination with antiserum against wild type *Campylobacter* and against flagella-less *Campylobacter*, and active vaccination with an inactivated wild type *Campylobacter* whole cell preparation.

Chickens

Four-days-old or two-weeks-old SPF chickens.

Experimental design experiment 1.

Four groups of ten 4-days-old chickens were (once daily) treated orally with 0.8 ml chicken antiserum to wild type *Campylobacter* strain 81116, or with 0.8 ml chicken antiserum to the flagellaless mutant 81116-R2 or with o.8 ml unvaccinated control chicken serum, or were left untreated as control. The first day (i.e. at 4-days of age) chicken received antisera just before challenge with 3.2 x 10^7 CFU/ml, as well as 6 hours after challenge. Treatments were continued until necropsy. At 5 or 10 days after challenge 5 birds of each group were killed and the CFU per gram cecal content was determined (see below: Post-mortem and bacteriology).

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Experimental design experiment 2.

Four groups of ten 4-days-old chickens were (once daily) treated orally with 0.8 ml chicken antiserum to wild type *Campylobacter* strain 81116, or with chicken antiserum to the flagellaless mutant strain 81116-R2 or with unvaccinated control chicken serum or were left untreated. The first day (i.e. at 4 days of age) antisera were given just before challenge with 1.4×10^8 CFU/ml, as well as 6 hours after challenge. Treatments were continued until necropsy. At 5 days after challenge the chickens were killed and the CFU per gram cecal content was determined.

Experimental design experiment 3.

Three groups of 10 two-weeks-old chickens were vaccinated IM with 1 ml of the whole cell vaccines in a Freunds Incomplete type of water in oil emulsion, containing inactivated cells of wild type *Campylobacter* strain 81116 or the flagellaless mutant strain 81116-R2 or were left unvaccinated. At 5 weeks of age all chickens were challenged orally with wild type *Campylobacter* strain 81116, 1.4×10^8 CFU/ml. One week after challenge the chickens were killed and the CFU per gram cecal content was determined.

Post-mortem and Bacteriology

Chickens were killed and the content of each caecum was gently removed, weighed and diluted to 0.1 g per ml in 0.04 M PBS. Serial 10-fold dilutions were then plated out on selective Blaser *Campylobacter* agar plates. After 48 hours of incubation at 41°C under microaerophilic conditions, the CFU per gram cecal content was determined.

RESULTS

Experiment 1

From Table 1 it can be concluded that repeated daily passive immunisation with serum against wild type *Campylobacter* or with unvaccinated chicken control serum had no effect on cecal colonisation by wild type *Campylobacter* if compared to untreated control chickens (at 5 days as w ll as 10 days after chall ng). All three groups showed high levels of cecal colonisation by *Campylobacter* (up to $> 10^8$ CFU per gram cecal

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content). In sharp contrast, passive immunisation with antis rum according to the in-vention (i.e. raised against a flagella-negative mutant) resulted in limination of wild type *Campylobacter* from the ceca (or prevented colonisation). A level of <3 means that the number of CFU / gram caecum content is below the level of detection.

Experiment 2

In this experiment testing was repeated (see Table 2). Again a strong reduction of ce-cal colonisation was found in chickens passively vaccinated with antiserum according to the invention (i.e. raised against a flagella-negative mutant): 6/10 chickens were completely negative and a mean reduction of > 3 logs was found.

Experiment 3

Because literature indicated that active immunisation with wild type *Campylobacter* could result in maximally 2 logs reduction of *Campylobacter* colonisation (Widders, P.R., Perry, R., Muir, W.I., Husband, A.J. and Long, K.A., 1996, Br. Poultry Sci. 37:765-778.), we tested and compared wild type and R2 based vaccines in an active protection model.

From the results (Table 3) it is clear that neither vaccine had an effect on cecal coloni-sation.

R isolation of C. jejuni 81116, 5 days after challenge

	Log CFU/gram caecum content			
Antiserum	C. jejuni 81116	C. jejuni R2	Not vaccinated	No antiserum
Chicken				
1	8.1	<3.0	7.8	7.6
2	5.9	<3.0	8.7	5.6
3	8.5	<3.0	8.1	9.1
4	5.5	<3.0	7.7	7.2
5	8.6	<3.0	7.6	8.8
Mean	7.3	<3.0	8.0	7.7
Sd	1.5		0.4	1.4

Table 1a.

Reisolation of C. jejuni 81116, 10 days after challenge

	Log CFU/gram caecum content			
Antiserum	C. jejuni 81116	C. jejuni R2	Not vaccinated	No antiserum
Chicken				
1	9.0	3.5	7.8	8.1
2	9.1	<3.0	8.9	7.6
3	9.0	<3.0	9.3	7.4
4	9.1	<3.0	7.0	7.8
5	8.5	<3.0	8.7	8.2
Mean	8.9	<3.1	8.3	7.8
Sd	0.3	0.2	0.9	0.3

Table 1b.

Reis lation of *C. jejuni* 81116, 5 days after challenge

Antiserum	Log CFU / gram caecum content			
Chicken	*C. jejuni* 81116	*C. jejuni* R2	Not vaccinated	No antiserum
1	7.5	3.8	9.5	<3.0
2	<3.0	9.1	7.9	7.5
3	7.4	8.9	7.6	7.9
4	7.5	6.0	8.4	8.5
5	6.0	<3.0	7.3	9.0
6	9.1	<3.0	7.0	5.8
7	8.9	<3.0	6.6	9.0
8	7.6	<3.0	9.1	7.0
9	<3.0	<3.0	7.5	9.0
10	<3.0	<3.0	9.4	9.8
Mean	<6.3[a]	<4.6[b]	8.0	<7.7
Sd	2.4	2.5	1.0	2.0

Table 2

[a] $p \leq 0.05$ compared to the group receiving antiserum of not vaccinated chickens (two sample t-tests)

[b] $p \leq 0.001$ compared to the group receiving antiserum of not vaccinated chickens (two sample t-tests)

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Reisolation of *C. jejuni* 81116, 6 days after challenge.

Chicken	Log CFU / gram caecum content		
	Vaccination with *C. jejuni* 81116	Vaccination with *C. jejuni* R2	Not vaccinated
1	8.3	5.2	8.3
2	8.7	8.0	8.7
3		8.7	
4	7.1	8.5	7.1
5	8.1	7.8	8.1
6	8.4		8.4
7	7.7	7.8	7.7
8	8.5	8.8	8.5
9	9.1	8.3	9.1
10	7.5	7.4	7.5
Mean	**8.2**	**7.8**	**8.2**
Std.	0.6	1.1	0.6

Table 3.

Conclusion:

Vaccines based upon antibodies raised against flagella-less *Campylobacter* are capable of eliminating wild-type *Campylobacter* from the caecum. This is in sharp contrast to their counterparts having antibodies raised against wild type *Campylobacter*.

It is also in sharp contrast to vaccines comprising wild type *Campylobacter* cells.

Claims

1) Vaccine for the prevention of *Campylobacter* colonisation in animals, characterised in that said vaccine comprises antiserum raised against a flagellaless *Campylobacter* strain.

2) Vaccine according to claim 1, characterised in that the flagellaless *Campylobacter* strain is *Campylobacter jejuni*, and the animals are poultry.

3) Vaccine according to claim 2, characterised in that the flagellaless *Campylobacter jejuni* strain is strain R2.

4) Antigenic protein having a molecular weight of 97 kD (+/- 5 kD), characterised in that it is visible in a Western blot of *Campylobacter jejuni* protein after incubation of said Western blot with antibodies against a flagellaless mutant of *Campylobacter jejuni* and that it is not visible after incubation of said blot with antibodies against wild type *Campylobacter jejuni*.

5) Antigenic protein having a molecular weight of 60 kD (+/- 5 kD), characterised in that it is visible in a Western blot of *Campylobacter jejuni* protein after incubation of said Western blot with antibodies against a flagellaless mutant of *Campylobacter jejuni* and that it is not visible after incubation of said blot with antibodies against wild type *Campylobacter jejuni*.

6) Vaccine for the prevention of *Campylobacter jejuni* colonisation in poultry, characterised in that said vaccine comprises antibodies against the antigenic protein according to claim 4 or 5.

7) Vaccine for the prevention of *Campylobacter jejuni* colonisation in poultry, characterised in that said vaccine comprises an antigenic protein according to claim 4 or 5.

8) Use of antibodies against a flagellaless *Campylobacter* strain, or against th protein of claim 4 or 5 for the preparation of a vaccine against *Campylobacter* colonisation in animals.

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9) M thod for the preparation of a vaccin according to claim 1-3 or 6, characterised in that said method comprises raising antibodies against antigenic material of a flagellaless *Campylobacter* strain in a host animal followed by isolating the antiserum from the host animal.

10) Method for the preparation of a vaccine according to claim 6, characterised in that said method comprises growing antibody producing cells and harvesting the antibodies.

11) Method for the preparation of a vaccine according to claim 7, characterised in that said method comprises admixing a protein of claim 4 and/or 5 and a pharmaceutically acceptable carrier.

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Abstract

The present invention relates to vaccines against *Campylobacter* colonisation in animals. The invention also relates to the use of *Campylobacter* proteins and antibodies raised against *Campylobacter* for the preparation of such vaccines. Finally, the invention relates to methods for the preparation of such vaccines.
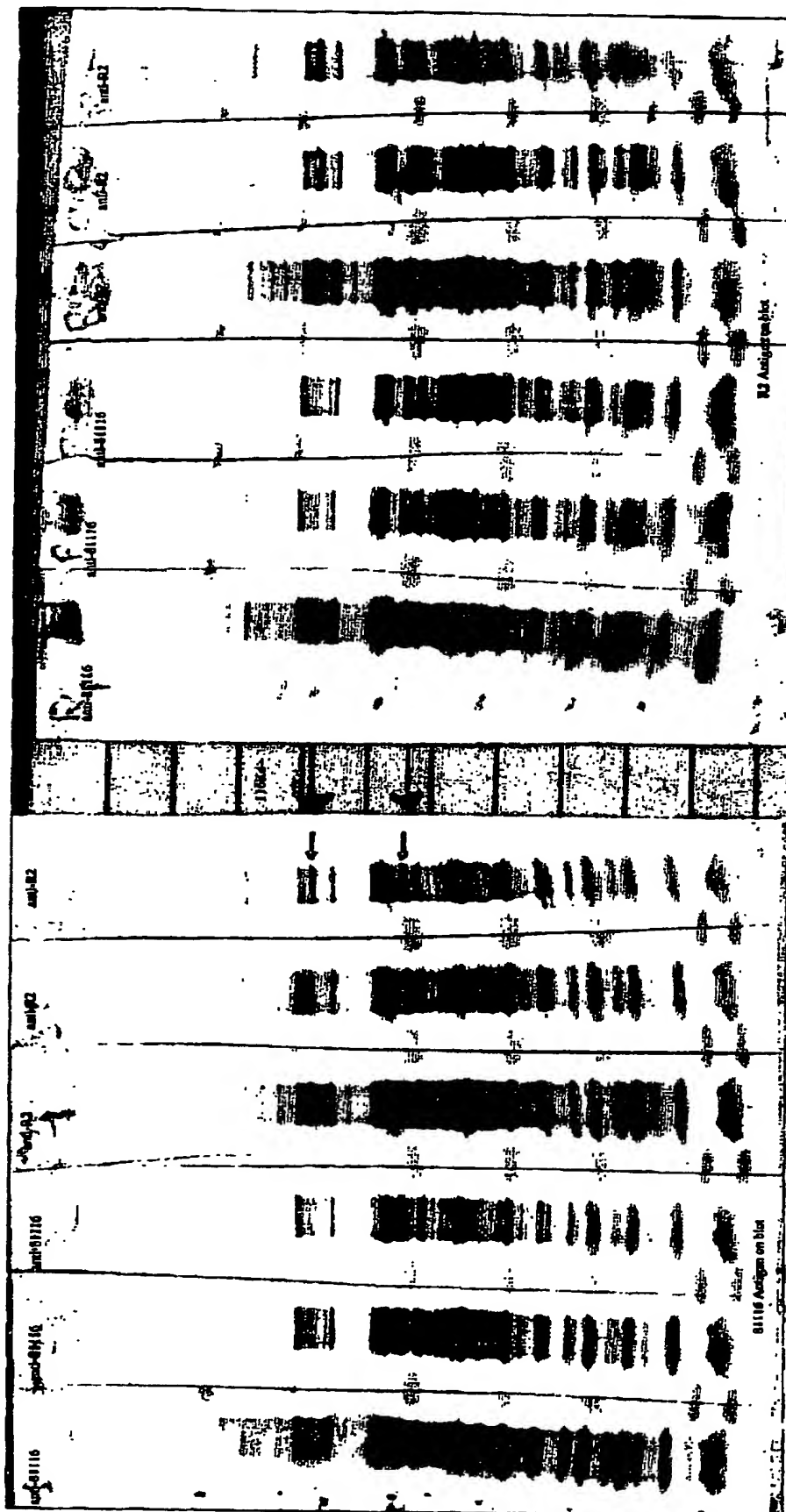
Figure 1